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(71) Applicant: LIGAND PHARMACEUTICALS, INCORPORATED [US/US]; 9393 Town Centre Drive, San Diego, CA 92121 (US).

(72) Inventors: McDONNELL, Ronald, P.; 10382 Rue Riviera Verte, San Diego, CA 92131 (US). TZUKERMAN, Maty; Kibbutz Lohamei, 25220 Hatettaot (IL).

(74) Agents: WARBURG, Richard, J. et al.; Lyon & Lyon, 34th floor, 611 West Sixth Street, Los Angeles, CA 90017 (US).

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(54) Title: METHOD FOR SCREENING FOR RECEPTOR AGONISTS

(57) Abstract

Method for assay for a receptor agonist. The method includes the steps of: providing nucleic acid encoding a receptor having a first TAF region able to activate transcription from a promotor, and a second TAF region mutated to have the functional context of the second TAF region, but not able on its own to activate transcription of the promoter. The nucleic acid is provided within a cell unable to exhibit transcription from the promoter in the presence of the second TAF region alone, but able to exhibit transcription from the promoter in the presence of the first TAF region. The cell further includes a reporter construct containing the promoter. The reporter construct is transcribed when the promoter is activated in the presence of the first TAF region. The method further includes contacting the cell with a potential agonist, under conditions in which contact of the cell with a known agonist of the receptor causes transcription from the promoter, and increases the level of the product of the reporter construct. Finally, the method involves measuring the level of increase of the product of the reporter construct as an indication of the agonist activity of the potential agonist.

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METHOD FOR SCREENING FOR RECEPTOR AGONISTS

BACKGROUND OF THE INVENTION

This invention relates to methods and constructs useful for identifying or screening agonists active at cell receptors, such as at hormone receptors, e.g., the estrogen receptor.

The following is a discussion of relevant art, none of which is admitted to be prior art to the claims.

Evans et al., U.S. Patent 5,071,773, (hereby incorporated by reference herein) describes an assay by which hormone receptors, ligands for such receptors, and proteins having transcription activating properties of a hormone receptor, can be detected. Generally, the assay involves use of a cell which contains both DNA encoding a hormone response element (e.g., a promoter) linked to an operative reporter gene, and DNA encoding a receptor protein. When a suitable hormone or ligand is provided to the cell, a hormone receptor - hormone complex is formed and delivered to an appropriate DNA-binding region to thereby activate the hormone response element and cause expression of the reporter gene. Activation of the reporter gene is detected by standard procedures used for detecting the product of the reporter gene.

Pierre Chambon and his group have described many properties of the estrogen receptor, and its alleged cell-type and promoter-context dependent activity. These experiments were generally performed by use of one or more truncated estrogen-receptor-encoding genes which express receptors lacking all or a portion of two domains termed TAF1 and TAF2. These domains are thought to be regulated by estrogen and then cause promoter activation. A third domain is located between these two, and is thought to bind to DNA near a promoter activated by the receptor-hormone complex (Kumar et al., 51 Cell 941 (1987)).

Specifically, Webster et al., 54 Cell 199 (1988), use chimeric receptors to localize regions responsible for transcription activation function. The authors propose that a hormone is responsible for allowing a receptor to recognize a DNA response element, and that the hormone induces a transcription activation function in the hormone-binding domain.

Tora et al., 59 Cell 477 (1989), analyzed truncated mutants of human estrogen receptor, and described TAF1 and TAF2 as two transcriptional activation functions in the receptor. These activators are said to exhibit cell-type specificity and promoter-context dependency. The authors indicate that TAF2 acts synergistically with upstream elements.

Meyer et al., 57 Cell 433 (1989), describe inhibition of transcription stimulation by the progesterone receptor by co-expression of the estrogen receptor. The authors propose that the observations reflect competition by the receptors for a limiting transcription factor.

Berry et al., 9 EMBO Journal 2811 (1990), describe so-called promoter- and cell-specific effects of an agonist on estrogen-responsive genes. Truncated and chimeric estrogen receptors were used which contained TAF1 and/or TAF2 regions from the same or different sources.

Tassett et al., 62 Cell 177 (1990), describe interaction of TAF1 and TAF2 regions, and competition (squelching) for limiting factors, by comparing relative activities of TAF regions and competitor constructs.

Fawell et al., 60 Cell 953 (1990), describe estrogen-receptor dimerization and its alteration by mutations in the molecule.

Metzger et al., 20 Nucleic Acids Research 2813 (1992), describe alleged promoter- and cell-specificity of TAF1 and TAF2 regions in the yeast *Saccharomyces*

cerevisiae. Truncated receptors, or receptors having regions deleted from them, were used in the analyses.

Danielian et al., 11 EMBO Journal 1025 (1992), describe conserved regions in the estrogen receptor and state that:

> "Activities of TAF1 and TAF2 vary depending upon the responsive promoter and cell type and, in some cases, both are required for full transcriptional stimulation."

The authors identify amino acids near the C-terminus of the mouse estrogen and glucocorticoid receptors which are said to be essential for hormone dependent stimulation of transcription. Point mutations were introduced either into the full-length receptor, or into an internal deletion mutant which lacked the TAF1 region, to allow the authors to determine the effects of mutations upon TAF2 activity in the absence or presence of TAF1.

SUMMARY OF THE INVENTION

Applicant has discovered that the activity of TAF1 and TAF2 regions in a receptor are interdependent. That is, the activity of one region is dependent on having the functional context of the other region available. (By "functional context" is meant that only a few amino acids (*i.e.*, up to 10), or preferably only ~~(i.e.~~ 1-3~~)~~ amino acids are altered in one region so that the interaction of a hormone or transcription factor with the region is altered to a minimum extent (preferably, the interaction is unaltered). Such interaction will allow full expression of the activity of the unaltered or non-mutated region. Thus, the functional context of one TAF region contains the functional activities of the other TAF region with respect to agonist binding, dimerization, and heat shock protein interaction, but not with respect to the ability

to activate transcription.) Applicant's discovery indicates that appropriate assays for detection of agonists of a chosen receptor require receptor constructs in which a minimum number of mutations are introduced into either the TAF1 or TAF2 regions to inactivate that region, i.e., to make it nonfunctional, without having significant effect on the activity of the other, non-mutated, region. Thus, in one example, mutations are introduced into the TAF2 region which make the TAF2 nonfunctional, while allowing TAF1 to exhibit its functional activity.

Unlike prior studies which used forms of TAF1 or TAF2 having deleted regions, assays of the present invention provide reproducible results which can be readily interpreted. Prior constructs failed to provide the information required to determine the agonist or antagonist activity of any chosen molecule at a selected receptor.

Specifically, applicant has discovered that it is possible to provide an assay to screen for an agonist at a receptor which interacts with only one of the TAF1 or TAF2 regions of the receptor. Such a specific assay for an agonist allows rapid screening of large numbers of agonists for those having specific and desired properties. For example, an agonist at the estrogen receptor can be readily identified, e.g., as one which has activity similar to estrogen or tamoxifen or other known agonists. That is, not only can an agonist be specifically identified, but the type of agonist can be determined in such an assay.

More specifically, applicant has identified methods by which a modified receptor can be used to identify promoter- and cell-type specific requirements for TAF1 or TAF2 activity of a receptor. Experiments can now be performed to determine such promoter- and cell-type specific differences in activity.

Without being bound to any specific theory, applicant proposes that such promoter- and cell-type specificity may be explained by a model in which the TAF1 region acts as a dominant transcriptional activator, and the TAF2 region as a transcriptional facilitator. That is, the TAF2 region acts to prepare the transcription apparatus for TAF1 action. Such preparation may be recruitment of basic transcription factors, alteration of chromatin structure, or causing removal of a transcriptional repressor. Alternatively, the TAF2 region may prepare a transcription apparatus for other transcriptional activators, and alone have little inherent transcription activation activity. In such a model, the TAF1 region is unable to access the transcription apparatus until the TAF2 region has acted appropriately to prepare it for TAF1 action.

Applicant proposes that cell specificity for TAF1 or TAF2 activity may reflect the presence or absence of a TAF1 or TAF2-type function in a cell that mimics the presence of TAF1 or TAF2, respectively. Such a mimetic in a cell will allow a receptor construct having a mutated and inactive TAF1 or TAF2 region, to be active. That is, the inactive portion of the receptor can be complemented by the active functionality present in the cell. A similar model may exist for promoter specificity, i.e., only selected promoters will be activated by TAF1 or TAF2 in any particular cell, dependent on the functionalities present in those promoters. In this model, the difference in agonist activity of various agonists is dependent on the effect of that agonist on the TAF1 region or TAF2 region, and interaction of the resulting TAF1 or TAF2 region with a selected promoter or general transcription apparatus.

Applicant has taken advantage of these findings to develop an assay by which agonists of receptors can be readily identified. In this method, a

cell is provided with a specific receptor construct having a selected TAF1 and/or TAF2 activity, and having a suitable response element (e.g., containing a promoter region) linked to an operative reporter gene (e.g., encoding an enzyme activity which is readily detectable). The response element is selected in conjunction with a specific cell so that activity of an agonist is observed only under selected conditions. Thus, in one example, the receptor may have an active TAF1 region, and a mutated (inactive) TAF2 region which provides the functional context of TAF2, and the cell is chosen such that it has a component which mimics or replaces the TAF2 region function of the receptor on the chosen promoter. The promoter in turn provides an appropriate binding context to allow the component to manifest the desired TAF functions. In this way, an agonist which acts at the TAF1 region can be readily identified by its ability to cause expression of the reporter gene, despite the lack of an active TAF2 region on the receptor.

Thus, in a first aspect, the invention features a method for screening or assaying for an agonist at a chosen receptor. The method includes providing a cell containing nucleic acid encoding a receptor having a TAF1 and a TAF2 region. One of these TAF1 and TAF2 regions is able to activate transcription from a selected promoter, and the other region is mutated so that, while it provides the functional context of that region, it is not able to activate transcription of the promoter independent of the other TAF region.

The cell is chosen such that no, or minimal, transcription of the promoter occurs in the presence of a receptor having only an unmutated TAF region corresponding to that mutated above (and not the other TAF region). The cell is also chosen such that

transcription occurs in the presence of a receptor having the above nonmutated region alone. For example, in a receptor construct having an operative TAF1 region and a mutated, inoperative TAF2 region, transcription of the promoter will not occur (i.e., no significant level of transcription is detectable, usually less than 5-10% of normal levels) in the presence of a receptor having an operative TAF2 region only, but will occur in the presence of a receptor having an operative TAF1 region only. As discussed above, applicant postulates that such a cell contains a factor which mimics the TAF2 function of the receptor.

The cell further includes a reporter construct which has a promoter region which is activated to cause transcription of a reporter gene in the presence of a receptor having an active TAF region corresponding to that which is not mutated above. The promoter is not activated by the presence of a receptor containing only the TAF region corresponding to that mutated above.

The method further includes the step of contacting the cell with a potential agonist under conditions in which contact of the cell with a normal agonist (e.g., estrogen for an estrogen receptor) will cause transcription from the promoter, and thereby increase the level of the reporter gene product. The method may involve transcribing the reporter construct at a basal (low or minimal) level in the cell before the agonist is applied. Alternatively, the method may involve applying the agonist first, and then transcribing the reporter construct. The receptor may contain two TAF regions. Non-limiting examples of receptors that may be used in the present invention include estrogen receptors, progesterone receptors, androgen receptors, or mutated versions of the above receptors.

Finally, the method involves the step of measuring the level of increase of the reporter gene product, as an indication of the agonist activity of the potential agonist.

In preferred embodiments, the agonist is a human hormone agonist, and a nuclear receptor, e.g., a human hormone receptor is encoded by the nucleic acid within the cell. In one example, the receptor has a mutated TAF2 region, and the cell and promoter are chosen to exhibit no, or minimal, response to the presence of TAF2. One example of such a cell is a liver cell (specifically, a HepG2 cell) in which a receptor with an operative TAF2 region has no activity. That is, there is no inherent transcriptional activity with a receptor having just TAF2 and no TAF1 region present in the cell, but there is transcriptional activity with a receptor having an operative TAF1 region alone available. Most preferably, the promoter is chosen such that it does not require a receptor with a TAF2 function to be provided within the chosen cell, so that any agonist which acts in conjunction with a functional TAF1 in the receptor construct is able to show its agonist activity.

In another aspect, the invention features a method for detection of agonist activity by provision of a cell having a nonmutated receptor having functional TAF1 and TAF2 regions. The cell is chosen to lack a mimicking TAF1 or TAF2 activity (i.e., a receptor having either an active TAF1 or TAF2 region, alone does not cause activation of transcription in the cell). The promoter is chosen so that activation is achieved in this cell from this promoter in the presence of an agonist for the receptor which acts only through one TAF region and not both. For example, a liver cell (e.g., HepG2) and a complex C3 promoter together provide a useful assay for agonists active at a TAF1 region only.

The liver cell and C3 promoter have a TAF2 activity, but the promoter is not activated in the presence of a receptor having an active TAF2 region alone. But, in the presence of an active TAF1 region, the promoter is active. Thus, agonists active at the receptor TAF1 region can be identified as those which cause expression of the reporter gene.

In a preferred embodiment, the method concerns use of a receptor in which the TAF2 region is mutated, and provided within the selected cell and promoter context. This cell provides a useful screening test to determine the type of agonist tested. The level of transcription observed is related to the agonist type as exemplified below. The above two methods (with mutated and nonmutated receptor constructs) may be used in combination to detect, and grade or type agonists at a selected receptor.

In another aspect, the invention features a method for treating or preventing an estrogen related disease or condition. By "estrogen related disease" is meant a disease that is caused or associated with an elevated or depressed level of the hormone estrogen. By "hormone" is meant a naturally occurring biochemical that will function as a receptor agonist. Synthetic hormones are more properly referred to as agonists. Examples of estrogen related diseases include osteoporosis, breast cancer, uterine cancer, and endometriosis. Examples of estrogen related conditions include vasomotor abnormalities, hot flashes, depression, other psychiatric abnormalities and uterine fibroids. In some diseases the patient may be unable to produce estrogen in an amount required by the body. In other diseases, estrogen may be overproduced. The treatment may have the effect of preventing new tumors from developing and/or of shrinking the size of existing tumors. The method includes forms of hormone

replacement therapy. In this method, the patient is preferably first identified as suffering from such a disease or condition by standard techniques, and then treated as described below.

The method involves administering a chemical compound other than keoxifene, having a keoxifene-like transcriptional profile to a patient (or causing production in vivo of such a compound). By "keoxifene-like transcriptional profile" is meant the production of a normalized response similar to keoxifene. This profile demonstrates a relatively low TAF1 response at low concentrations of the compound but relatively high response at higher concentrations of the compound. In addition, little or no TAF2 response should be present at all concentrations. In addition, it should have a greater (about twice or more) TAF1 response than with a wild type receptor at higher concentrations (about 10^{-7}M), see Fig. 8E compared to Figs. 8A-D). The administration of these chemical compounds with keoxifene-like transcriptional profiles is expected to exhibit bone protecting activity and uterine/breast sparing activity. By "bone protecting activity" is meant the ability to prevent bone resorption which can be measured by standard techniques. Bone resorption is typically associated with a loss of estrogen. Bone resorption is typically associated with osteoporosis and is manifest by bone dissolution due to a loss of calcium. By uterine/breast sparing activity" is meant the prevention or reduction of the proliferation of tumorous cancer cells which can be measured by standard techniques.

Examples of compounds that can be screened to determine whether or not they have a keoxifene-like transcriptional profile are provided in Jones, U.S. Patent No. 4,418,068, issued November 29, 1983 and incorporated herein by reference. As the Jones patent

makes clear, other compounds that could be screened include dihydronaphthalenes and benzothiophenes.

Other compounds that can be screened include compounds with a similar chemical structure to keoxifene or keoxifene-like analogs. Some of these compounds could be produced by making substitutions of 1-10 carbon long alkyl, alkenyl or similar-type chains in the nitrogen-containing ring of keoxifene. Other alterations could include altering the length or saturation characteristic of the alkyl chain (e.g., from 0-10 carbon atoms) that links the nitrogen-containing ring to the rest of the keoxifene compound. Other compounds that can be screened for a keoxifene-like profile include compounds with a chemical structure similar to tamoxifine or tamoxifine analogs. Those skilled in the art will readily recognize other modifications and substitutions that can be made to compounds that can be screened for a keoxifene-like profile.

The present invention also includes pharmaceutically acceptable compositions prepared for storage and subsequent administration which include a pharmaceutically effective amount of an above-described product in a pharmaceutically acceptable carrier or diluent.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The drawings will first briefly be described.

Drawings

Fig. 1. Transcription Activation by ER-wt (wild type estrogen receptor) and Truncated ER Mutants.

Schematic organization of ER-wt, ERN282G and ER179C (A). CV1 (B), HepG2 (C) and HS578T (D) cells,

were transiently co-transfected with increasing concentrations of the different receptor expression vectors as indicated, together with 9.5 μg/ml of ERE-tk-LUC reporter plasmid, and 5 μg/ml of pRSV-β-gal expression vector as an internal control for transfection efficiency. Carrier DNA (pGEM4) was added to adjust the total amount of DNA to 20 μg/ml (see below). Cultures were treated with or without 10^{-7}M 17-β-estradiol (E_2) as indicated for 36 hours and assayed for β-galactosidase and luciferase activity (LUC activity is normalized for β-gal activity). The relative luciferase activity is calculated by dividing the normalized luciferase value at a given point by that obtained in the absence of transfected receptor or ligand. A single experiment representative of four independent experiments is detailed above. Data shown indicate the mean ± SE(m) of triplicate estimations.

Fig. 2. Transcription Activation by Mutant ER Defective in TAF2 Activity.

Schematic organization of ER-wt and mutant ERs used in this experiment (A). CV1 (B), HepG2 (C) and HS578T (D) cells were transiently co-transfected with increasing concentrations of different receptor expression vectors as indicated, together with 9.5 μg/ml of ERE-tk-LUC reporter plasmid, 5 μg of pRSV-β-gal expression vector. Carrier DNA was added to 20 μg total DNA. Cultures were treated with or without 10^{-7}M 17-β-estradiol (E_2) for 36 hours, and assayed for luciferase and β-gal activity. The relative luciferase activity is calculated by dividing the normalized luciferase value at a given point by that obtained in the absence of transfected receptor or ligand. A single experiment representative of four independent experiments is detailed above. Data shown indicates the mean ± SE(m) of triplicate estimations.

Fig. 3. Activity of TAF1 and TAF2 on the Human C3 Gene Promoter.

CV1 (A), HepG2 (B) and HS578T (C) cells were transiently co-transfected with 0.5 μg of the indicated receptor expression vector, 9.5 μg of C3-LUC reporter plasmid, 5 μg pRSV-β-gal and carrier DNA to a total amount of 20 μg DNA. In addition, a minus receptor control was included. Cultures were treated with or without 10^{-7}M 17-β-estradiol (E_2) for 36 hours, and assayed for luciferase activity. The data shown are representative curves of experiments that have been repeated 6 times with similar results. The curves represent averages of quadruplicate data points averaged and normalized for transfection efficiency by simultaneous estimation of pRSV-β-gal transcriptional activity.

Fig. 4. Activity of ER-TAF1 and ER179C on Different Promoter Constructs.

CV1 (A) and HepG2 (B) cells were transiently co-transfected with 0.5 μg of the indicated receptor expression vector, 9.5 μg of pA_2-LUC reporter plasmid, 5 μg pRSV-β-gal and carrier DNA to a total amount of 20 μg. CV1 (C) and HepG2 (D) cells were co-transfected as described above, using the pEREMLT-LUC reporter. Cultures were treated with or without 10^{-7}M β-estradiol (E_2) for 36 hours and assayed for luciferase activity. Data presentation is described in Fig. 1.

Fig. 5. Activation of ER-TAF1 and ER179C by Triphenylethylene-derived Estrogen Partial Agonists.

HepG2 cells were co-transfected with 0.5 μg of the indicated receptor expression vectors, 9.5 μg of C3-LUC reporter, 5 μg of pRSV-β-gal and carrier DNA to a total amount of 20 μg. Cultures were treated with 10^{-7}M of 17-β-estradiol (A), E_2, Tamoxifen (B), 4-hydroxy-Tamoxifen (C), Nafoxidine (D), Clomiphene (E), for 36 hours and assayed for luciferase activity. Data

presentation was described in Fig. 1. In Fig. 5, ER represents Er-wt, ER^{m} represents Er-TAF1, TAF2 represents Er-179c, and TAF2^{m} represents Er-Null.

Fig. 6. Displacement of Estradiol Binding to ER-wt and ER-TAF1 Proteins by Estrogen Agonists.

Yeast cytosols prepared from cell expressing ER-wt or ER-TAF1 were incubated overnight at 4°C with 5 nM of ^{3}H-17-β-estradiol alone or in the presence of the indicated concentrations of the different estrogen agonists. Ligand binding was determined by scintillation counting following separation of bound and free ligand using hydroxylapatite.

Fig. 7. Model for TAF1 and TAF2 as Functionally Dependent Activators of Transcription.

This schematic outlines a hypothesis for the promoter and cell specificity of the individual transactivators of the estrogen receptor. Interaction of the receptor with ligand initiates a cascade of events which exposes the receptor DNA binding region (DBD) and promotes association of ER with DNA. Only "estrogenic compounds" are capable of functionally activating TAF2 region of the receptor. Upon activation (B), the TAF2 region of the receptor interacts with a transcriptional repressor (I), displacing it or altering its structure (C) to permit the TAF1 activation sequence access to the general transcription apparatus (GTA). In certain cells and on certain promoters, TAF2 function of the receptor can be supplied by other transcription factors, allowing TAF1 region of the receptor to function independently of TAF2. Therefore, binding of the receptor to DNA is synonymous with transactivation and can be accomplished by both estrogen agonists, as well as antagonists that permit delivery of the receptor to DNA. In this model, the partial agonist activity of the triphenylethylene-derived estrogen agonists depends on the conformation induced by the ligand and the effect

that this conformation has on the presentation of TAF1 to the transcription apparatus.

Fig. 8 shows that the partial agonist activities of the triphenylethylene derived antiestrogens depends on TAF1 function. HepG2 cells were cotransfected with 0.5 μg of the indicated receptor expression vectors, 9.5 μg of C3-LUC reporter, 5 μg of pRSV-β-gal and pGem4 as carrier DNA to a total amount of 20 μg. Cultures were treated with various concentrations of 17-β-estradiol (A), Clomiphene (B), Nafoxidine (C), 4-OH-Tamoxifen (D) and keoxifene (E), for 36 hours and assayed for luciferase activity. The data for panel E was obtained relative to a different estradiol control than the other panels. Thus, the peak in panel E appears approximately five times higher than it would if the data had been obtained relative to the same estradiol control that was used in panel A. The relative luciferase activity was calculated as described for Fig. 1. A single experiment representative of 6 independent experiments is detailed. The data shown indicate the mean ± SE(m) of triplicate estimations.

Fig. 9. Bone marrow from sham (control), OVX (ovariectomized rats), OVX plus estrogen, and OVX plus keoxifene treated rats were evaluated for osteoclastic potential in the coculture assay. Bone marrow was combined with primary osteoblasts in the presence of 1,25-dihydroxyvitamin D3 and parathyroid hormone for 8 days and scored for the number of tartrate acid phosphatase resistant multinucleated cells (TRAP + MNC). The number of TRAP + MNC in the sham operated animals was set at 100%.

Fig 10. Estrogen agonist activity of keoxifene (keox) on MCF-7 cell proliferation. The activity of estrogen in this assay is maximum at 10^{-10}M, and induces proliferation to 1500% of control.

Fig. 11 is a diagram showing pC3-LUC.

Methods

The methods discussed briefly above are useful for identifying agonists of various receptors. For example, an estrogen agonist can be identified which is useful for treatment of osteoporosis. In the disease state, it appears as though TAF1 activity alone is sufficient for prevention of bone loss. Thus, agonists having activity only at the TAF1 region and not at the TAF2 region of the receptor are useful for disease treatment. The methods described herein allow rapid screening of potential agonists, unlike prior methods in which laborious procedures were involved to detect useful agonists. Other useful receptors for which this procedure can be used includes progesterone, glucocorticoid, androgen and mineralocorticoid receptors, not only in the human derived cells, but also in other eucaryotic cell lines, such as chicken and yeast (which are useful organisms for screens of the present invention).

The following are specific examples of methods of this invention. These examples make use of the estrogen receptor, but are not limiting in the invention. Those in the art will recognize that other equivalent receptors, cells and promoters can be readily used in equivalent procedures within the scope of the claims.

Estrogen Receptor

The estrogen receptor (ER) is a member of the nuclear receptor super-family, a class of transcription factors whose functions are regulated by steroids, vitamins or thyroid hormone (Beato, 56 Cell 335, 1989). This family of regulatory proteins share common mechanistic features in that they are transcriptionally inactive within the cell until exposed to hormone. Occupancy by hormone results in transformation of the receptor to an activated state, thus allowing it to

productively interact with specific DNA sequences in the regulatory regions of target genes. The resultant positive or negative effects of the bound receptor on specific gene transcription are cell-type and promoter-context dependent. Nonetheless, the relative effect may be measured in any particular cell/promoter construct. Thus, the desired effect may be observed in a wide variety of constructs.

The cDNA for ER has been cloned and used to reconstitute estrogen responsive transcription units in heterologous mammalian cells (Kumar et al., 5 EMBO J. 2231, 1986, and Green et al., 231 Science 1150, 1986). This has enabled a detailed examination of the functional domains within the protein (Kumar et al., 51 Cell 941, 1987). A functional examination of the domains of ER in several systems has revealed the likely structural features within the receptor which may interface with critical cellular components to generate a variety of hormone responsive endpoints (Danielian et al., 11 EMBO J. 1025, 1992). In particular, two distinct transactivation domains have been defined, a sequence at the amino terminus of the receptor, termed TAF1, and a sequence confined to the carboxyl 60 amino acids, termed TAF2 (Danielian et al., supra; Berry et al., 9 EMBO J. 2811, 1990; and Tasset et al., 62 Cell 1177, 1990), all hereby incorporated by reference herein. Recently, investigators involved in intracellular receptor research have favored referring to the TAF domains, as AF domains (e.g. Cavailles et al., J. of Cellular Bio., 341, 1994) to avoid confusion with the discovery and cloning of TATA-binding protein associated factors (Dynlacht et al., 66 Cell, 563, 1991).

It appears that alterations in charged residues of the amino terminal portion of the hormone binding domain can result in increases or decreases in

ER transcriptional activity with no change in receptor affinity of cognate hormone. Therefore, the regions around residue 530 (Danielian et al., supra,) and the region around cysteine 381 (Pakdel et al., 7 Mol Endocrinol, 1408, 1993) may in themselves consititute AF subdomains within TAF-2. It follows then, that changes in such domains, such as the region around cysteine 381, could result in mutant receptors which could discriminate between estrogen and antiestrogen ligands, paralleling the results obtained as detailed herein. Futhermore the analogous situation could exist for discreet residues in the TAF-1 region of ER.

The cellular targets of ER-TAF1 and ER-TAF2 have not yet been identified. A rigorous examination of ER-TAF1 and ER-TAF2 function in mammalian cells has not been yet accomplished.

Examples

The following experiments characterize the dependence of ER-TAF1 and ER-TAF2 activities on cell-and promoter-context in mammalian cells, and the role of ligand (agonist) in manifestation of these differences. Some of these experiments are described in Tzukerman et al., 8 Mol. Endocrin 21, 1994, hereby incorporated by reference herein.

The following materials and methods were used in the example:

Receptor expression vectors

cDNA sequences encoding the ER-wt and a TAF1 - deleted receptor derivative were excised from the plasmids YEpwtER and YEpER179C respectively, using BfrI and SacI. The DNA encoding the TAF1 receptor derivative was excised from the plasmid YePERN282G using BfrI and KpnI. Construction of the vectors YEpwtER, YEpER179C and YEpERN282G, have been described previously (Pham et al., 6 Mol. Endo. 1043, 1992). The excised DNA was treated with T4 DNA polymerase (Boehringer Mannheim Co.)

and ligated into the unique EcoRV site within the mammalian expression vector pRST7 (Berger et al., 41 J. Steroid Biochem. Mol. Biol. 733, 1992).

Receptor mutations

The wild type estrogen receptor cDNA was cloned into pGEM-11Zf(+) (Promega, Wisconsin). Specific mutations were introduced into the hormone binding domain of the receptor by substituting alanine for amino acids located at positions 538, 542, and 545, using site directed mutagenesis (Kunkel et al., 154 Methods in Enzymology 367, 1987), creating the plasmid pGERm. The mutated hormone binding domains were introduced into ER-wt and ER179C by exchanging the BglII-SacI C-terminal fragment of this vector with the analogous mutated fragment from pGERm.

Reporter plasmids

The reporter ERE-tk-LUC contains a single copy of the vitellogenin ERE upstream of the herpes simplex thymidine kinase promoter sequences linked to luciferase (LUC). The C3-LUC reporter which contains 1.8 kb of the human C3 gene promoter (-1807 to +58) (Vik et al., 30 Biochemistry 1080, 1991). Unique restriction sites Xho1 and BamH1 were introduced into the C3 promoter, the DNA was then cloned into the cognate sites of the vector pl-LUC (Berger et al., 41 J. Steroid Biochem. Mol. Biol. 733, 1992), where a polyclonal site has been inserted into the MMTV-LUC vector (see Fig. 11). Those in the art can readily construct equivalent vectors. pA2-LUC contains a 835 bp fragment (-821 to +14) of the Xenopus vitellogenin A2 gene promoter (Vik et al., 30 Biochemistry 1080, 1991). pEREMLT-LUC contains a single ERE upstream the adenovirus major late promoter sequences (-44 to +11) (Hu and Manly, 78 Proc. Natl. Acad. Sci. USA 820, 1981).

Cell culture

CV1 and HS578T cells were routinely maintained in Dulbecco's modified Eagle's medium - DMEM (Biowittaker, Maryland) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Utah). HepG2 cells were maintained in Minimal Essential Medium Eagle's - MEM (Biowittaker, Maryland) containing 10% FCS.

Transient transfection assay

Cells were seeded 24 hours prior to transfection in flat-bottom 96-well tissue culture plates ($5x10^3$ cells/well), in phenol red-free DMEM containing 10% FBS. DNA was introduced into cells using calcium phosphate ~~co~~-precipitation. Plasmid DNA was diluted in 1 ml of 1 mM Tris, pH 7.4, 0.1 mM EDTA, 0.25 M $CaCl_2$. DNA solution was added dropwise with vortexing into an equal volume of 2X HBS pH 6.9 (280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4) and precipitates were allowed to form for 20 minutes. Transfections (11 μl of DNA mix/well) were performed on a Biomek 1000 Automated Laboratory Workstation (Beckman, California). Cells were transfected for 6 hours and then washed with phosphate-buffered saline (PBS) to remove the precipitate. Cells were incubated for an additional 36 hours in phenol red-free medium containing 10% charcoal-treated FBS, with or without hormones as indicated in the text. Cell extracts were prepared as described by Berger et al., 41 J. Steroid Biochem. Mol. Biol. 733, 1992, and assayed for luciferase and β-galactosidase activities. All determinations were performed in triplicate in at least two independent experiments, and were normalized for transfection efficiency by using expression of β-galactosidase as an internal control.

Preparation of yeast receptor proteins

Expression vectors producing ER-TAF1 were constructed by replacing the Bfr1-Mlu1 fragment of

YEpE10 (Pham et al., 88 Proc. Natl. Acad. Sci. USA 3125, 1991) with the corresponding fragment of pRST7ER-TAF1. This vector and a vector producing wild type receptor (YEPE10) were transformed into the yeast strain BJ2168 (previously described by McDonnell et al., 39 J. Steroid Biochem. Molec. Biol. 291, 1991). Individual transformants were picked and grown to an OD_{600}=1. Cultures were then induced with 100 μM $CuSO_4$, and 2 mM chloroquine for 16 hours at 30°C. Cells were then pelleted and washed with cold water. Cells were resuspended in 2-5X pellet volume of 10 mM Tris, 0.4 M KCl, 2 mM EDTA, 0.5 mM PMSF, 1 μg/ml aprotinin, 2 mM DTT, pH 7.6, and lysed by vortexing with 0.45-0.5 mm glass beads, intermittently with cooling on ice, until at least 90% of the cells were observed to be open. Extracts were centrifuged at 13,000xg and the supernatants were recovered. Protein concentrations were determined by Bio-Rad Protein Assay (Bio-Rad, Richmond, CA).

β-Estradiol binding competition assay

All methods were performed using a Biomek 1000 automated workstation (Beckman Instrument, Fullerton, CA). Ten-fold serial dilutions of the compounds to be tested were made in 10 mM Tris, 0.3 M KCl, 5 mM DTT, pH 7.6, and transferred to polystyrene tubes containing 100 μl at final concentrations of 10^{-4} M to 10^{-11} M diluted compounds, 5 mM ^{3}H-β-estradiol (Amersham, UK), and 22 μg total protein derived from strains producing ER or ER-TAF1. Following an overnight incubation at 4°C, 100 μl of a 6% hydroxylapatite slurry in 10 mM Tris, 5 mM DTT, pH 7.6 was added. The tubes were incubated for an additional 30 minutes at 4°C, mixing after the first 15 minutes. Hydroxylapatite pellets were washed 4X with 1 ml 1% Triton X-100 in 10 mM Tris, 5 mM DTT, pH 7.6. Finally, the hydroxylapatite pellets were resuspended in 800 μl of Ecoscint A scintillation fluid

(National Diagnostics, Manville, NJ). Activity in each sample was measured using a LS6000IC scintillation counter (Beckman Instruments, Fullerton, CA).

Example 1: Transcriptional Activation by TAF1 and TAF2 Truncated Receptors

Referring to Fig. 2, truncated forms of the human estrogen receptor were prepared which lacked either the TAF1 (ER179C, see Fig. 2A) or the TAF2 (ERN282G, see Fig. 2A) activation sequence. These constructs encode proteins structurally similar to those used previously in mammalian (Berry et al., 9 EMBO J. 2811, 1990) and yeast cells (Pham et al., 6 Mol. Endo. 1043, 1992). The transcriptional activities of these ER derivatives were assessed using a reporter plasmid containing one copy of the vitellogenin estrogen response element (ERE) (Klein-Hitpass et al., 76 Cell 1053, 1986) inserted upstream of the thymidine kinase promoter (ERE-tk-LUC). The reporter plasmid and increasing concentrations of ER or mutant ER expression vectors were transiently transfected into the ER negative cell lines CV-1 (monkey kidney fibroblasts), HepG2 (human hepatocellular carcinoma) and HS578T (human breast cancer cells), and activity assessed as documented in Fig. 2B. All transfections were performed in the absence or in the presence of 17-β-estradiol at concentrations ranging from 10^{-5}M to 10^{-11}M. However, due to the number of data points obtained in this way (>2,500) only the activities obtained using 10^{-7}M 17-β-estradiol are presented since this is a concentration that elicited maximal transcriptional response in all cell lines examined.

The ER-wt was active in all cell lines. Using this protocol, we were unable to detect significant TAF1- mediated transcriptional activity in either CV-1, HepG2, HS578T (Figs. 2B, C, D) or HeLa or U2OS cells (data not shown) when assayed in the context of the

ERN282G deletion. In contrast, however, the TAF2 activation function (ER179C) exhibited substantial activity in these cells (Figs. 2B, C & D). The magnitude of the TAF2 transcriptional activity by ER179C was cell-type dependent. This isolated activator exhibited a lower efficacy relative to wild type receptor, even at DNA concentrations that produced saturating receptor levels. In HepG2 cells ER179C was about 35% as active as ER-wt (Fig. 2C), whereas in CV-1 and HS578T, the ER179C demonstrated 70% and 65% of ER-wt activity respectively (Figs. 2B & D). Transfection efficiency and recombinant expression levels were similar as estimated by indirect fluorescence microscopy and flow cytometric analysis (data not shown).

The results obtained in this analysis are consistent with the hypothesis that the TAF1 and TAF2 sequences represent functionally distinct transcriptional activators. A wild type receptor activity requires either both activator regions or an intact receptor context for an individual activator to exhibit maximal transcriptional activity.

In addition to the partial activities observed by the above ER-mutants, increasing concentrations of transfected ER-wt in CV-1 and HS578T cells led to a progressive decrease in hormone dependent transcriptional activity (Figs. 2B & D). This phenomenon has been observed by others and likely results from sequestration of limited transcription factors or targets by the over-expressed, hormone-activated receptor, such that activated receptor function is compromised (Tasset et al., 62 Cell 1177, 1990). This "squelching" or "transcriptional interference" supports the idea that ER requires additional, limiting cellular transcription factors for appropriate function. The failure of the ER-wt to "squelch" in the HepG2 cell line (Fig. 2C) suggests

either an increased abundance of a required co-factor, or the involvement of an additional cell specific component in this process.

Example 2: Transcription Activation by a Mutant ER Defective in TAF2 Activity

Previously, TAF1 and TAF2 functions were defined as individual domains within the estrogen receptor that were capable of supporting transcription of an ER responsive promoter (Berry et al., 9 EMBO J. 2811, 1990, and Tasset et al., 62 Cell 1177, 1990). In the mammalian cells tested here, we were unable to show a distinct activity of the TAF1 sequence when analyzed in the context of the ERN282G deletion. We considered, therefore, whether analysis of this transactivator outside the context of the full-length receptor may not reflect its true biological activity. Previously, Danielian et al., supra, demonstrated that it was possible to change three amino acids between residues 535 and 550 in the carboxyl terminus of the mouse estrogen receptor which comprises transcriptional activity of TAF2, but nevertheless results in a receptor capable of binding both specific DNA and cognate ligand with wild type affinity, indicating that these changes did not lead to gross structural abnormalities in the protein. Therefore, using site-directed mutagenesis we created similar amino acid changes in the carboxyl terminus of human ER at residues 538, 542 and 549 (Danielian et al., supra). (see the ER-TAF1 construct of Fig. 1) This triple mutation was also introduced into ER179C creating a null estrogen receptor. (See the ER-Null construct of Figure 1) This latter construct allowed a specific determination of the effect of these mutations on TAF2 function. Thus, ER-Null and ER-TAF1 both serve as excellent controls because they are inactivated for the functions being studied. One of these constructs was used in every experiment documented

herein. Mutation of these three amino acids provides but one example by which the context of a TAF region can be maintained while inactivating that region. Those in the art will recognize that equivalent mutations in the same or other amino acids can be readily made by standard techniques.

The transcriptional activities of these mutant ERs were assessed by transient transfection into CV-1 cells together with the ERE-tk-LUC reporter. Introduction of the triple mutation into ER179C totally abolished TAF2 activity (Fig. 1B). Thus, we believed that introduction of this mutation into the wild-type ER, would allow an examination of TAF1 activity in the full-length receptor context without interference from TAF2 activity. The ability to specifically mutate the TAF2 activator within the human estrogen receptor in this manner is consistent with the results previously reported for the mouse ER, and indicates that equivalent mutations can be made in the other receptor TAF regions.

The full-length receptor containing the triple mutation (ER-TAF1) was subsequently used for analysis of TAF1 function in the context of the intact receptor. Constructs encoding wild type receptor, ER-TAF1, ER179C or the null estrogen receptor were transfected into CV-1, HepG2 or HS578T cells, together with the ERE-tk-LUC reporter. The results are shown in Fig. 1. In all cell lines, the ER179C was transcriptionally active, as observed earlier (Figs. 1B, C & D), whereas the null receptor was inactive. Interestingly, in CV-1 cells, in the absence of a functional TAF2 activation sequence, the ER-TAF1 protein exhibited a significant transcriptional activity (Figs. 1B, C & D). Thus, the activity of the TAF1 activator when analyzed in the context of a full length receptor molecule, as observed here, was different from that when analyzed as a deletion mutant (ERN282G, Fig. 2). This suggests that

TAF1 activity does not function independently, but rather requires additional carboxyl-terminal sequences for appropriate function. Interestingly, increasing the concentration of transfected ER-TAF1 DNA did not result in a receptor dependent "squelching" of transcriptional activity. This observation implies that both TAF1 and TAF2 activators and possibly the context of the full-length receptor are required for this squelching function.

A comparison was made of the expression level of each of these receptors by transfecting the expression vectors into CV-1 cells and analyzing the hormone binding activities in the resulting cytosolic extracts (data not shown). The K_ds of the ER-wt, ER-TAF1 and ER179C were the same. The ER-wt and ER-TAF1 were synthesized in comparable levels as measured by hormone binding activity, whereas the amino-terminally deleted ER179C and the null receptor were expressed at about 25% of ER-wt level. Since, all the transcriptional responses we detected with each receptor were maximal responses achievable, it is unlikely that receptor expression levels are a significant factor in the outcome of our experiments.

Example 3: ER-TAF1 and ER179C Activity is Promoter Specific

The above results using the ERE-tk-LUC reporter indicated that the TAF1 activator of the estrogen receptor functions, albeit weakly, in the absence of an intact TAF2 function. In addition, TAF1 activity appeared to be cell-type dependent.

We extended our studies to examine the efficacy of the individual activator functions on other estrogen responsive promoters. To this end, we chose the estrogen responsive C3 promoter in which a strong ERE has recently been identified (Zawaz, 2 Gene Exp., 39, 1992) (Vik et al. 30 Biochemistry, 1080, 1991). The

activities of the of ER-wt, ER-TAF1 and ER179C activators were evaluated on C3 promoter-directed transcription as depicted in Fig. 3. In HS578T cells, the C3 promoter can be activated equally well by either ER-wt, ER-TAF1 or ER179C (Fig. 3C). In contrast however, in HepG2 cells, the ER-TAF1 activator was as active in transcription as wild type ER, but the ER179C activator was silent (Fig. 3B). These data suggest that, with respect to the C3 promoter, there is a strong cell-type bias in ER transactivator functions. In CV-1 cells it appears that the combination of the activation sequences is required for maximal activity (Fig. 3A). Cumulatively, these data suggest that the TAF1 and TAF2 activators within ER demonstrate a dependence upon cell-type and promoter, and furthermore, the dominant activator of ER-mediated regulation of C3 expression is TAF1.

The analysis of the relative contribution of the individual ER TAF domains in ER function was extended to include two additional promoters, namely the adenovirus major late promoter, containing an estrogen response element, and the vitellogenin promoter (Fig. 4). Both of these promoters were responsive to estrogen in the presence of ER-wt. However, unlike the C3 promoter the individual activation domains of ER were minimally active in both cell lines examined. This highlights further the promoter specificity of the estrogen receptor activation domains. Similar tests to those described above can be used to quickly identify useful promoter and cell combinations for use in assays for agonists discussed above (see also, Example 4, below).

Example 4: Regulation of ER-TAF1 and ER179C Activity by Estrogen Receptor Agonists

Certain triphenylethylene-derived estrogen receptor antagonists (i.e., tamoxifen, nafoxidine) are

reported to exhibit partial agonist activities. We therefore tested whether these compounds preferentially activate either TAF1 or TAF2 transactivators. A series of these compounds was evaluated in HepG2 cells using the ER-TAF-specific receptor derivatives and the C3 promoter. On this promoter, tamoxifen, 4-hydroxy-tamoxifen, nafoxidine and clomiphene were all potent activators of ER-wt mediated C3 gene transcription (Figs. 5B-E). However, none of these compounds were as effective as estrogen in this regard (Fig. 5A). In addition, estrogen was an efficient activator of the ER-TAF1, whereas the partial estrogen agonists were not as effective. In this cellular and promoter context, ER179C was not activated by either estradiol or the partial estrogen agonists. These data imply that even though TAF1 activity is necessary, it alone does not activate this promoter by triphenylethylene-derived antihormones, suggesting that their mode of action may be mechanistically different from that of estrogen (Figs. 5A-F). The differences in hormonal responsiveness of these receptor derivatives do not relate to alterations in the affinity of the proteins for ligands. As shown in Figs. 6A and B, the affinity and specificity for ligands of the ER-wt and ER-TAF1 were indistinguishable. It is notable that not all the anti-hormones tested in this system have an identical transcriptional profile (Figs. 5B-E). The absolute efficacy for each of the anti-hormones is different, as is their ability to differentially activate TAF1, suggesting subtle mechanistic differences in the agonistic properties of these ligands.

These examples demonstrate that introduction of specific point mutations into the human estrogen receptor, affects ER-transcription activation function. Mutation of TAF2 in this manner is still compatible with wild-type binding affinities for estrogen, tamoxifen and

4-hydroxy-tamoxifen. Surprisingly, TAF1 retained considerable transcriptional activity despite TAF2 mutation. When we deleted the entire TAF2 sequence (ERN282G) we were unable to observe residual transcriptional activity of TAF1 in any cell line examined. This suggests that either TAF2 or the context of the full length receptor is required for full manifestation of TAF1 activity. In contrast, Berry et al. observed that a construct identical to ERN282G was constitutively active in avian CEF cells (Berry et al., 9 EMBO J. 2811, 1990). This may suggest a difference in estrogen receptor function in mammalian and avian cells, and may not reflect basic differences between the two sets of results.

Using the modified receptors we were able to identify cell and promoter specific differences in the activity of ER-TAF1 and ER179C. In studies which were controlled for expression level and transfection efficiency we saw that both activators displayed promoter and cell type specific differences in their activity. The most striking example of this is the inability of ER179C to function well on any promoter in HepG2 cells. The ER-TAF1 activator, on the other hand, functions very well on the complex C3 promoter, but less well on the other promoters examined. The activity profiles of ER-TAF1 and ER179C are clearly distinct, suggesting dissimilar mechanisms of action. On the complex C3 promoter there is no apparent synergism between TAF1 and TAF2, whereas it clearly exists on other promoters. This suggests that on this promoter the ER activation domains may interact with differing transcription factors. The data obtained using the C3 promoter in HepG2 cells indicate that there is a transcription factor in these cells that can functionally replace TAF2, as TAF1 is as good a transcriptional activator as ER-wt. However, since TAF2

alone does not activate transcription, it suggests that no mimetic for TAF1 exists for transcription of this promoter in this particular cell line.

The dissimilar mechanism of action and the promoter and cell type specificity can be explained by a model in which TAF1 is the dominant transcriptional activator and TAF2 is a transcriptional facilitator (see model Fig. 7). We suggest that the function of TAF2 is to "prepare" the transcription apparatus for TAF1 function. This "preparation" function could be recruitment of basic transcription factors, alteration of chromatin structure or overcoming the effects of a transcriptional repressor. On the other hand, TAF2 could "prepare" the transcription apparatus for another transcriptional activator, but on its own would have little inherent transcriptional activity. In support of this hypothesis, the TAF2 activator is poorly active on minimal promoters.

This dependence on promoter complexity is also observed for ER-TAF1 activity. Additional evidence in support of the facilitator role of TAF2 is that in yeast the TAF1 activator is inactive on a minimal promoter. However, a mutation of the SSN6 locus (a cellular repressor of transcription), Keleher et al., 68 Cell, 709, 1992, results in a 100-fold increase in ER-TAF1 activity (to a level comparable to ER-wt), whereas ER179C activity is not effected (McDonnell et al., 89 Proc. Natl. Acad. Sci. USA 10563, 1992). We suggest that this cellular mutation has the effect of mimicking the function of TAF2, and that in mammalian cells TAF2 has a similar role. In this system TAF1 is unable to access the transcription apparatus as a result of stearic hindrance by an inhibitor. Where TAF2 is available, then the inhibitor is displaced and TAF1 is able to interact with the transcriptional apparatus.

Example 5: Screening For and Use Of Compounds With Keoxifene Like Transcriptional Profiles

In humans the tri-phenylethylene derived anti-estrogen keoxifene, exhibits bone sparing activity while having no significant effects on uterine proliferation. In contrast, tamoxifen, a related anti-estrogen, is bone sparing but functions as a partial estrogen agonist in the uterus promoting an undesirable proliferative effect. In order to determine whether the differences in the in vivo biological activity of tamoxifen and keoxifene could be reconciled by their differential ability to transcriptionally activate TAF1, these compounds were studied in HepG2 cells using the ER-TAF specific receptor derivatives on the C3 promoter. The results are shown in Figure 8D and 8E.

Keoxifene had a unique transcription profile in this promoter and cellular context. In particular, low concentrations of keoxifene stimulated ER transcriptional activity. At higher concentrations, keoxifene inhibited the basal transcriptional activity of ER and did not cause further transcriptional activation (See Fig. 8E). On the ER-TAF1 construct, keoxifene demonstrated significant partial agonist activity inducing C3 promoter transcription 5-fold over background. The mechanism by which keoxifene manifests a transcriptional profile distinct from the related molecule tamoxifen is unclear. However, it is likely that these compounds induce subtle alterations in receptor structure which facilitate distinct interactions of the ER-TAFs with the general transcription apparatus.

The unique profile exhibited by keoxifene in this in vitro assay suggested that additional compounds displaying similar transcriptional profiles may also exhibit favorable bone protective/uterine sparing activities. To this end we have studied several

compounds derived from tamoxifen using this assay system and have been able to split these compounds into three distinct groups based on their ability to modulate TAF1 activity. The first category contains compounds that resemble the activity of estrogen, the second group resembles the activity of tamoxifen and the third group profile similar to keoxifene.

When the keoxifene-like compounds are assayed in the ovariectomized rat model they are expected to be bone protective and to demonstrate no uterotrophic activity. Rats are given a dorsal ovariectomy as follows. The animals are anesthetized with Ketamine:Xylazine and surgery is performed. Shave the central back of the anesthetized rat. Make a longitudinal incision in the skin parallel to the spine about 1 inch long. Spread the connective tissue away from the muscle layer with the tips of scissors. About 1 inch from the spinal column at the base of the rib cage, make a small cut (1/4") of the muscle with scissors. With small forceps, pull out ovarian fat. Ovary will be visible as a cluster-like structure, attached to the end of the uterine horn. Cut the connective tissue that holds them together. Staunch any bleeding. Replace fat into body cavity. Repeat on opposite side. Clip skin together and use betadine on the incision to retard infection and reduce clip removal. The next day injections are begun. Injections are done subcutaneously in the hip daily (usually in the morning). Vehicle is 10% ethanol and all injection volumes are 300 µl. After 28 days of injections, the animals are sacrificed under anesthetic by cervical dislocation and the body and wet uterine weights are determined. The hindlimbs are taken for histology and histomorphometry.

The transcriptional profile exhibited by keoxifene in vitro is predictive of agents demonstrating

bone selective estrogenic activity. Thus, the use of these ER-TAF constructs examined on this promoter and cellular context provides a useful screen for compounds useful for the treatment of osteoporosis.

In in vivo studies, rats are subjected to sham or authentic ovariectomy and allowed to recover for 5 days. Rats (4-6 per group) are then injected subcutaneously with vehicle or vehicle containing estrogen, keoxifene, or a test compound daily for periods up to 28 days. Animals are sacrificed, weighed, and evaluated for uterine wet weight, total serum cholesterol, and bone mineral density. Established methods are utilized with the exception that bone mineral density of the distal femural metaphysis is determined utilizing an Hologic mineral densitometer. Bone marrow from test animals is evaluated for osteoclastic potential in the coculture assay with primary osteoblasts. Bone marrow is combined with primary osteoblasts in the presence of 1,25-dihydroxyvitamin D3 and parathyroid hormone for 8 days and scored for the number of tartrate acid phosphatase resistant multinucleated cells (TRAP + MNC). The number of TRAP + MNC in the sham operated animals is set at 100%. Tartrate resistant acid phosphatase positive, multinucleated cells are scored by standard methods as nascent osteoclasts.

The in vitro effects of compounds on MCF-7 breast cell proliferation can also be studied. The partial agonist activities of estrogen, keoxifene, and the test compound on MCF-7 human breast carcinoma cells, is assessed by treating the cells for 7 days in the absence or presence of increasing concentrations of compound. Cells are treated at day 0 and day 4 with compound. Triplicate wells are evaluated for cell number at termination of the experiment on day 7. The activity of estrogen in this assay is expected to be

maximum at 10^{-10}M, and induce proliferation to 1500% of control.

The in vitro profiles of keoxifene and the test compounds could then be determined. The activities of increasing concentrations of compound on ER, ER-TAF1, and ER-null receptor transactivation of the C3 promoter in HepG2 cells is determined by standard methods as described in this document. Thus, compounds that exhibit both bone protecting and uterine/breast sparing activity at a given concentration, can be identified. Higher potency compounds than keoxifene are preferred. By "potency" is meant the amount of a compound required to produce the desired effect. Thus, high potency compounds will bind to the receptor with greater affinity than keoxifene. High potency compounds produce maximal effect at minimal dosages. As illustrated in Fig. 8, compounds with high potency have peaks further to the right than do compounds of lower potency.

Use

Agonists and their type can be quickly identified in the above systems. Specifically, the experiment described in Example 4, and illustrated in Fig. 5, is useful to identify an agonist and then define its type of activity. For example, use of a wild type receptor (ER-wt) in this assay will indicate whether the test compound is an agonist, i.e., has activity in the assay. The use of a mutated receptor with full functional context (ERm), in the assay will indicate the type of agonist, i.e., what level of activity is observed. Examples of the range of results expected with various test compounds are shown in Fig. 5, and discussed in Example 4. Using such assays, one can readily screen for desired agonist activity, e.g., agonists active only at TAF1 regions which mimic the

activity of estrogen and are useful for treatment of osteoporosis.

Pharmaceutical Compositions

The present invention also encompasses pharmaceutical compositions prepared for storage and subsequent administration, which have a pharmaceutically effective amount of the products disclosed above in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A.R. Gennaro edit. 1985). Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. For example, sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid may be added as preservatives. Id. at 1449. In addition, antioxidants and suspending agents may be used. Id.

The compositions of the present invention may be formulated and used as tablets, capsules or elixirs for oral administration; suppositories for rectal administration; sterile solutions, suspensions for injectable administration; and the like. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Suitable excipients are, for example, water, saline, dextrose, mannitol, lactose, lecithin, albumin, sodium glutamate, cysteine hydrochloride, and the like. In addition, if desired, the injectable pharmaceutical compositions may contain minor amounts of nontoxic auxiliary substances, such as wetting agents, pH buffering agents, and the like. If desired, absorption enhancing preparations (e.g., liposomes) may be utilized.

The pharmaceutically effective amount of the composition required as a dose will depend on the route of administration, the type of animal being treated, and the physical characteristics of the specific animal under consideration. The dose can be tailored to achieve optimal efficacy but will depend on such factors as weight, diet, concurrent medication and other factors which those skilled in the medical arts will recognize.

In practicing the methods of the invention, the products or compositions can be used alone or in combination with one another, or in combination with other therapeutic or diagnostic agents. These products can be utilized in vivo, ordinarily in a mammal, preferably in a human, or in vitro. In employing them in vivo, the products or compositions can be administered to the mammal in a variety of ways, including parenterally, intravenously, subcutaneously, intramuscularly, colonically, rectally, nasally or intraperitoneally, employing a variety of dosage forms.

As will be readily apparent to one skilled in the art, the useful in vivo dosage to be administered and the particular mode of administration will vary depending upon the age, weight and mammalian species treated, the particular compounds employed, and the specific use for which these compounds are employed. The determination of effective dosage levels, that is the dosage levels necessary to achieve the desired result, will be within the ambit of one skilled in the art. Typically, human clinical applications of products are commenced at lower dosage levels, with dosage level being increased until the desired effect is achieved. In non-human animal studies, applications of products are commenced at higher dosage levels, with dosage being decreased until the desired effect is no longer achieved or adverse side effects disappear.

The dosage for the products of the present invention can range broadly depending upon the desired affects and the therapeutic indication. Typically, dosages may be between about 10 μg/kg and 100 mg/kg body weight, preferably between about 100 μg/kg and 10 mg/kg body weight. Administration is preferably oral on a daily basis.

Other embodiments are within the following claims.

WE CLAIM:

1. Method for assay for a receptor agonist comprising the steps of:

providing nucleic acid encoding a receptor having a first TAF region able to activate transcription from a promoter, and a second TAF region mutated to have the functional context of said second TAF region but not able to activate transcription of said promoter wherein said nucleic acid is provided within a cell unable to exhibit transcription from said promoter in the presence of a receptor having said second TAF region alone, but able to exhibit transcription from said promoter in the presence of a receptor having said first TAF region, said cell further comprising a reporter construct comprising said promoter, said reporter construct being transcribed when said promoter is activated in the presence of a receptor having said first TAF region;

contacting said cell with a potential agonist, under conditions in which contact of said cell with a known agonist of said receptor causes transcription from said promoter, and increases the level of the product of said reporter construct; and

measuring the level of increase of said product of said reporter construct as an indication of the agonist activity of said potential agonist.

2. The method of claim 1, wherein said agonist is a human hormone agonist.

3. The method of claim 1, wherein said receptor has a mutated TAF2 region.

4. The method of claim 3, wherein said cell is a liver cell.

5. The method of claim 4, wherein said promoter is the C3 promoter.

6. The method of claim 1, wherein said assay provides a compound other than keoxifene with a keoxifene-like transcriptional profile.

7. Method for assay for a receptor agonist, comprising the steps of:

providing nucleic acid encoding a receptor having a functional first and second TAF region within a cell, wherein a receptor having only a functional TAF1 or TAF2 region fails to cause transcription from a promoter in said cell, said cell further comprising a reporter construct comprising a promoter which is activated in the presence of an agonist that acts only through one said TAF region but not both said TAF regions,

contacting said cell with a potential agonist, under conditions in which contact of said cell with a known agonist of said receptor causes transcription from said promoter, and increases the level of the product of said reporter construct; and

measuring the level of increase of said product of said reporter construct as an indication of the agonist activity of said potential agonist.

8. The method of claim 7, wherein said cell is a liver cell, and said promotor is a C3 promotor.

9. The method of claim 7, wherein said assay provides a compound other than keoxifene with a keoxifene-like transcriptional profile.

10. A method for treating a patient with an estrogen related disease comprising administering to

said patient a chemical compound other than keoxifene having a keoxifene like transcriptional profile.

11. The method of claim 10, wherein said disease is osteoporosis.

12. The method of claim 10, wherein said disease is uterine cancer.

13. The method of claim 10, wherein said disease is breast cancer.

14. The method of claim 10, wherein said treating comprises administering a pharmaceutically acceptable amount of said compound to said patient.

15. The method of claim 14, wherein said administering is performed orally.

16. The method of claim 10, wherein said compound has greater potency than keoxifene.

FIG. 1a.

FIG. 1b.

FIG. 1c.

FIG. 1d.

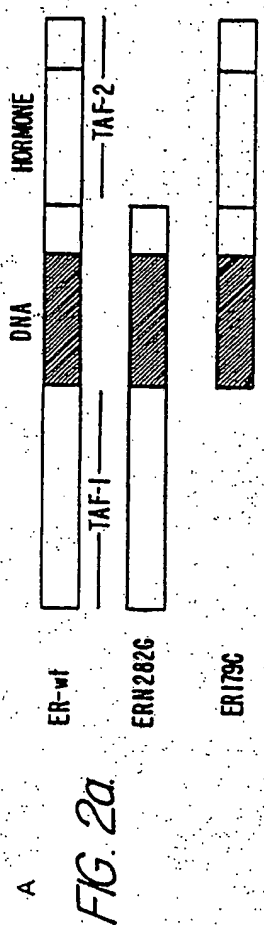


FIG. 2a

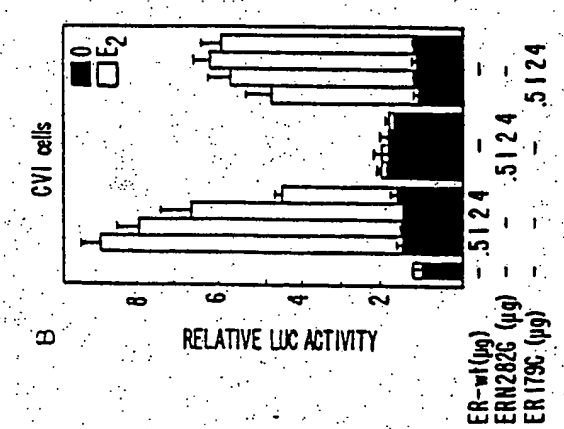


FIG. 2b

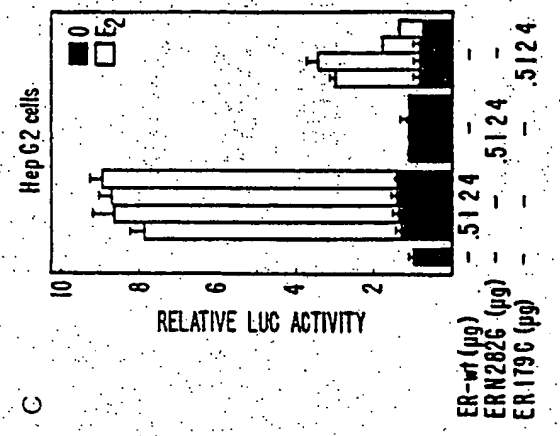


FIG. 2c

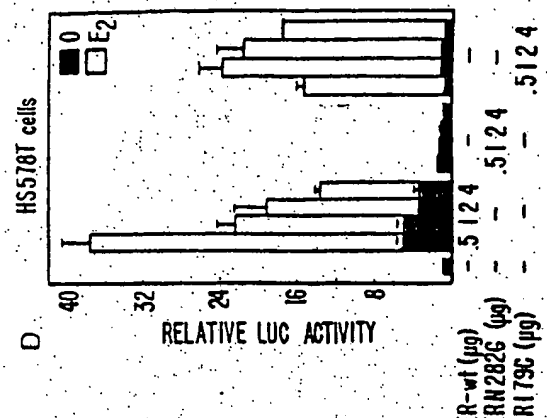


FIG. 2d

FIG. 3c.

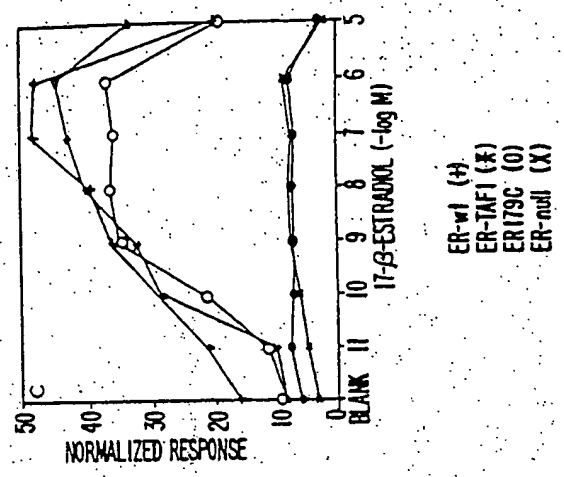


FIG. 3b.

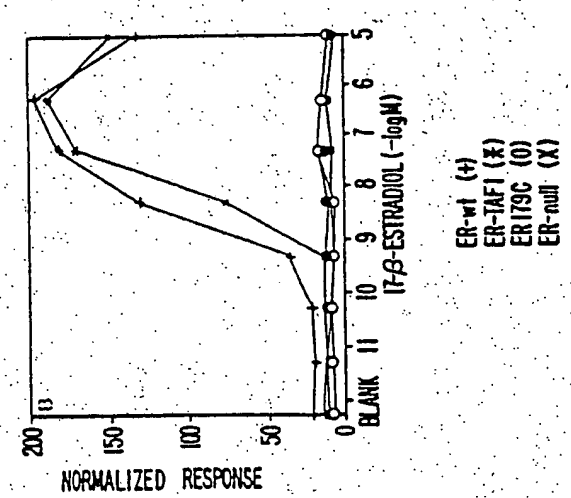


FIG. 3a.

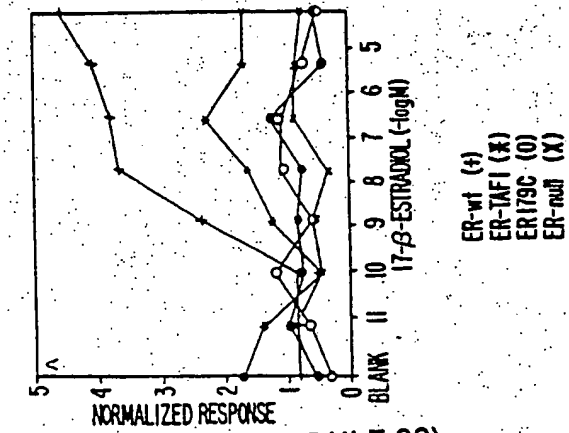


FIG. 4a.

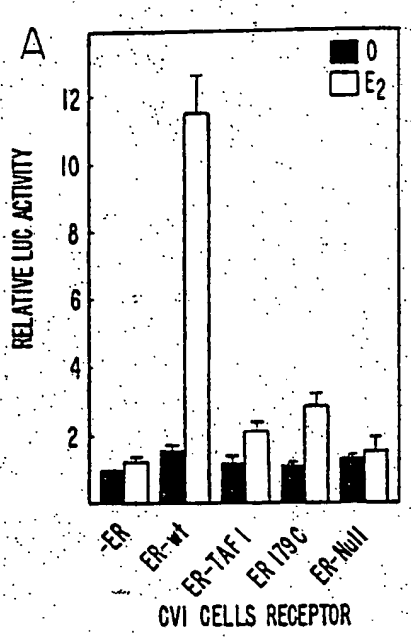


FIG. 4b.

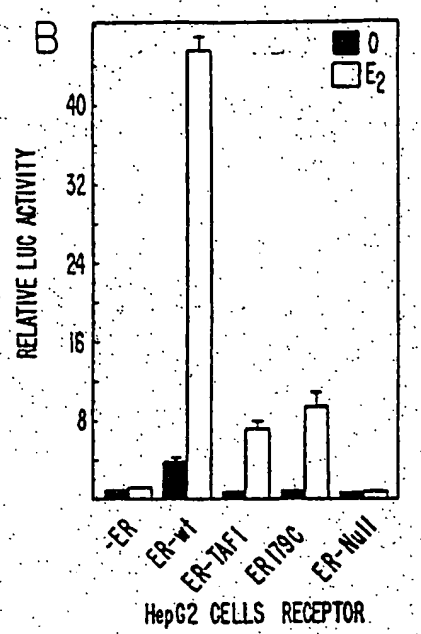


FIG. 4c.

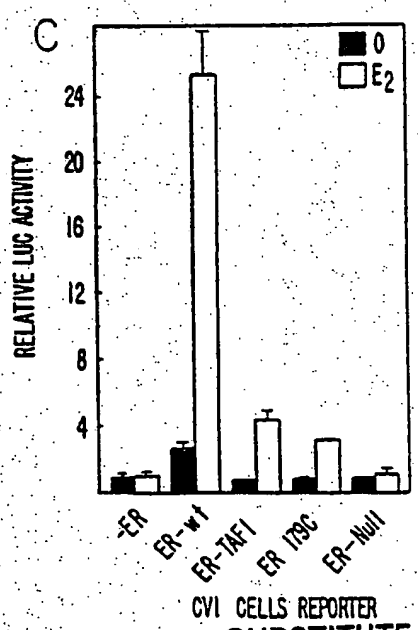


FIG. 4d.

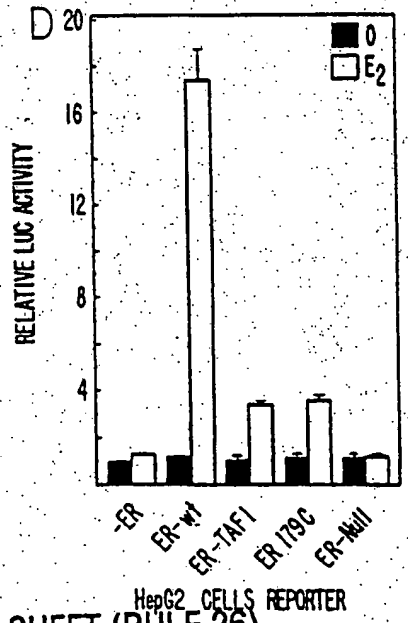


FIG. 5a.

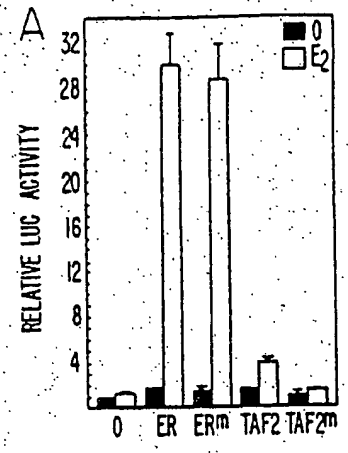


FIG. 5b.

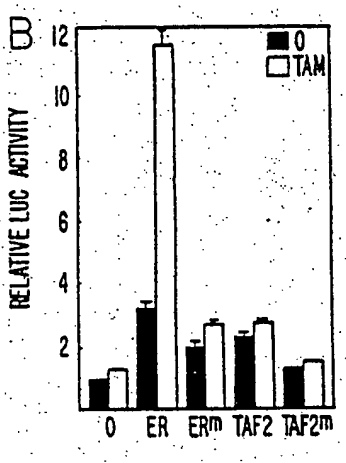


FIG. 5c.

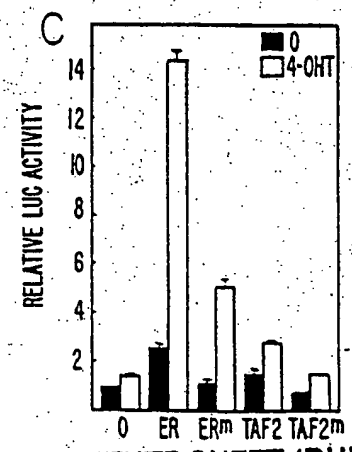


FIG. 5d.

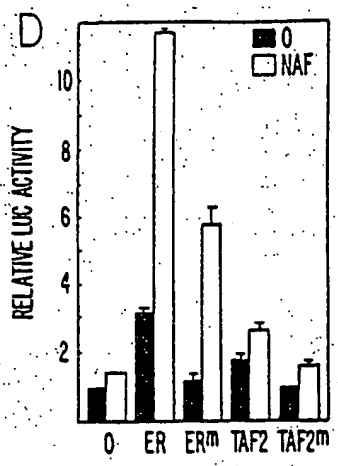


FIG. 5e.

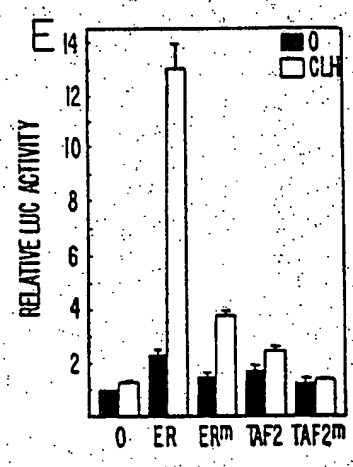


FIG. 5f.

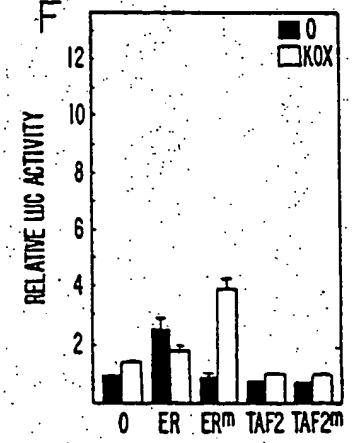


FIG. 6b.

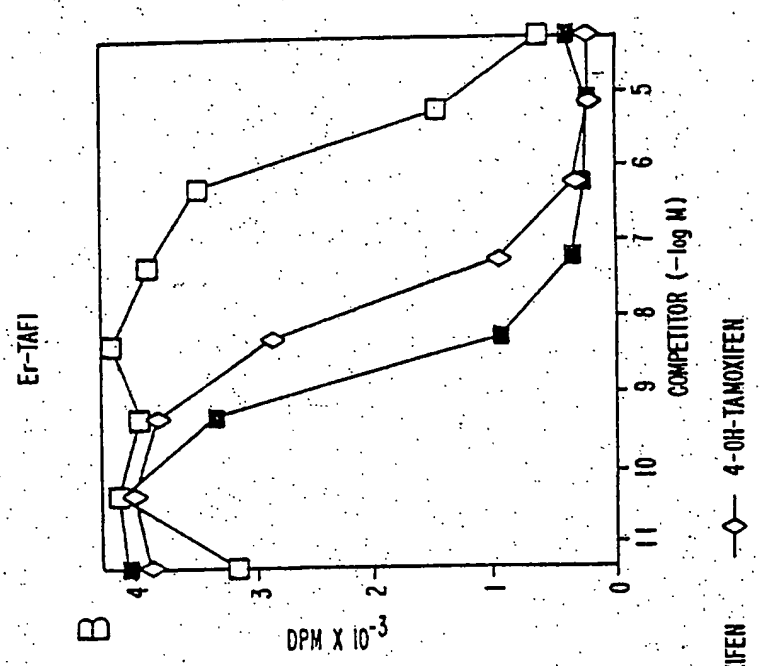

Er-TAF1
B
DPM X 10^{-3}
COMPETITOR (-log M)
4-OH-TAMOXIFEN

FIG. 6a.

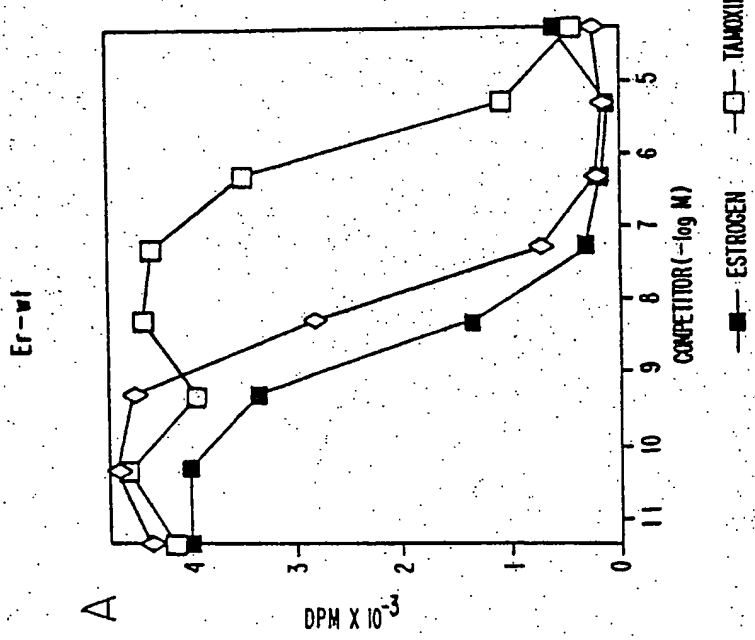

Er-wt
A
DPM X 10^{-3}
COMPETITOR(-log M)
ESTROGEN
TAMOXIFEN

FIG. 7c.

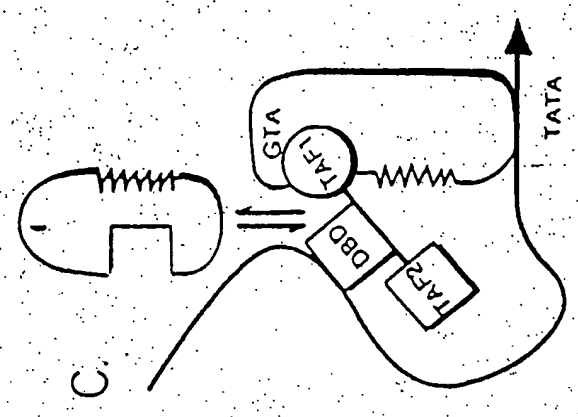


FIG. 7b.

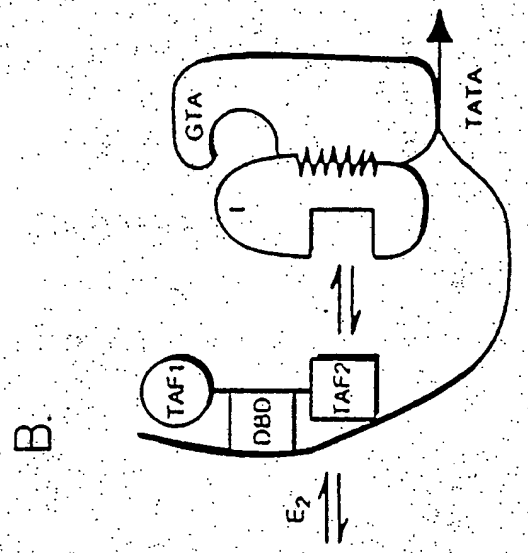


FIG. 7a.

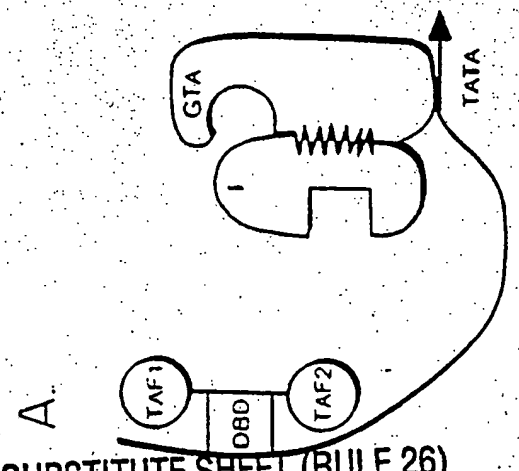


FIG. 8a.

A.

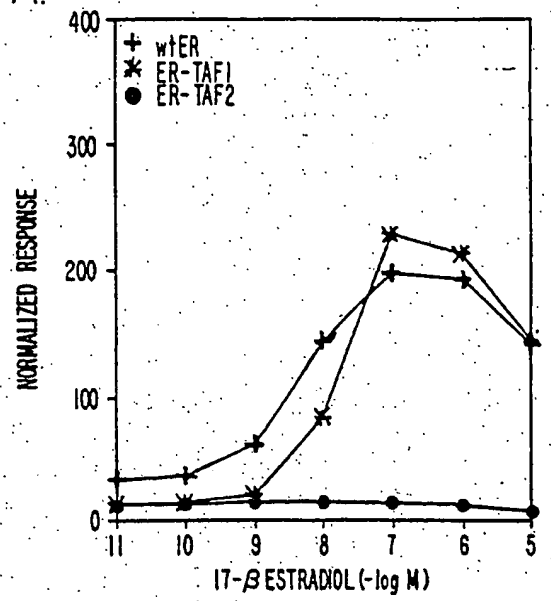


FIG. 8b.

B.

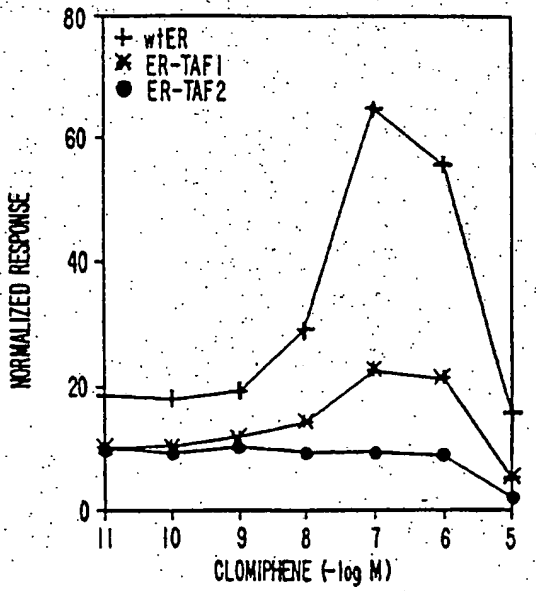


FIG. 8c.

C.

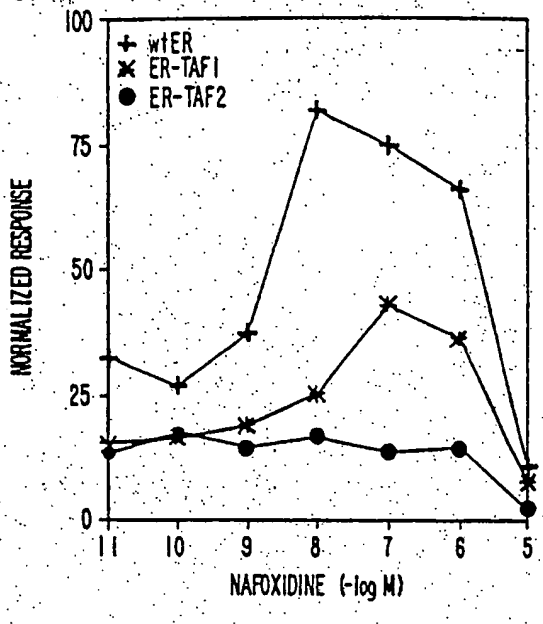


FIG. 8d.

D.

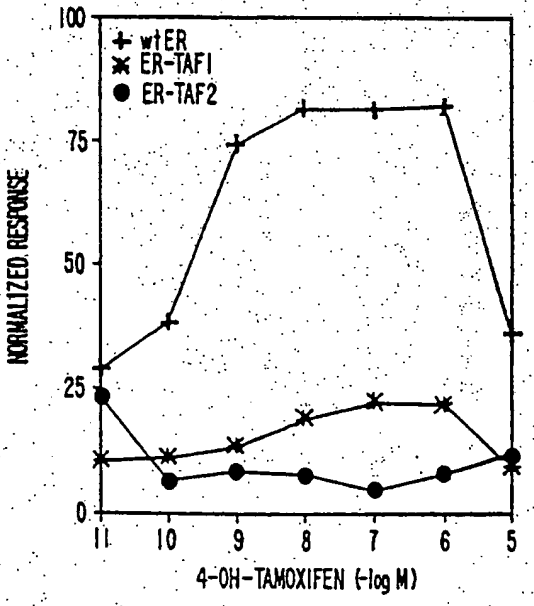


FIG. 8e.

E.

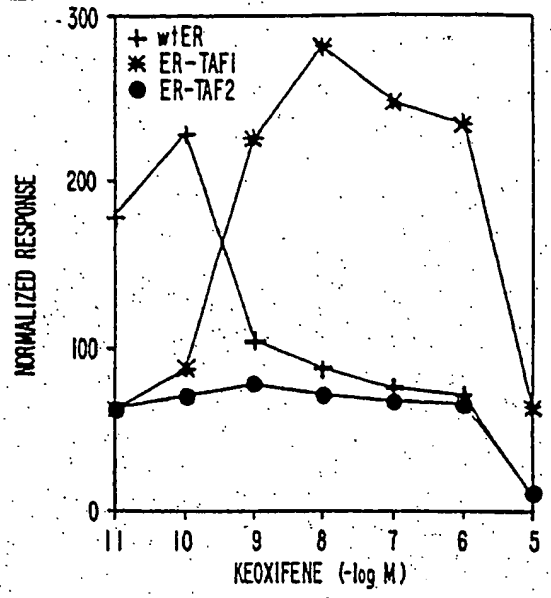


FIG. 9.

TRAP (+) MNC
(% CONTROL)

	Sham	OVX	OVX +17β E_2	OVX +Keoxifen
Expt. 1	100	147.8[a]	84.3[c]	99.3[c]
Expt. 2	100	154.0[b]	119.4[c]	78.2[a]

[a]SIGNIFICANTLY DIFFERENT FROM SHAM, $p<0.02$.

[b]SIGNIFICANTLY DIFFERENT FROM SHAM, $p<0.001$.

[c]NO SIGNIFICANT DIFFERENCE FROM SHAM.

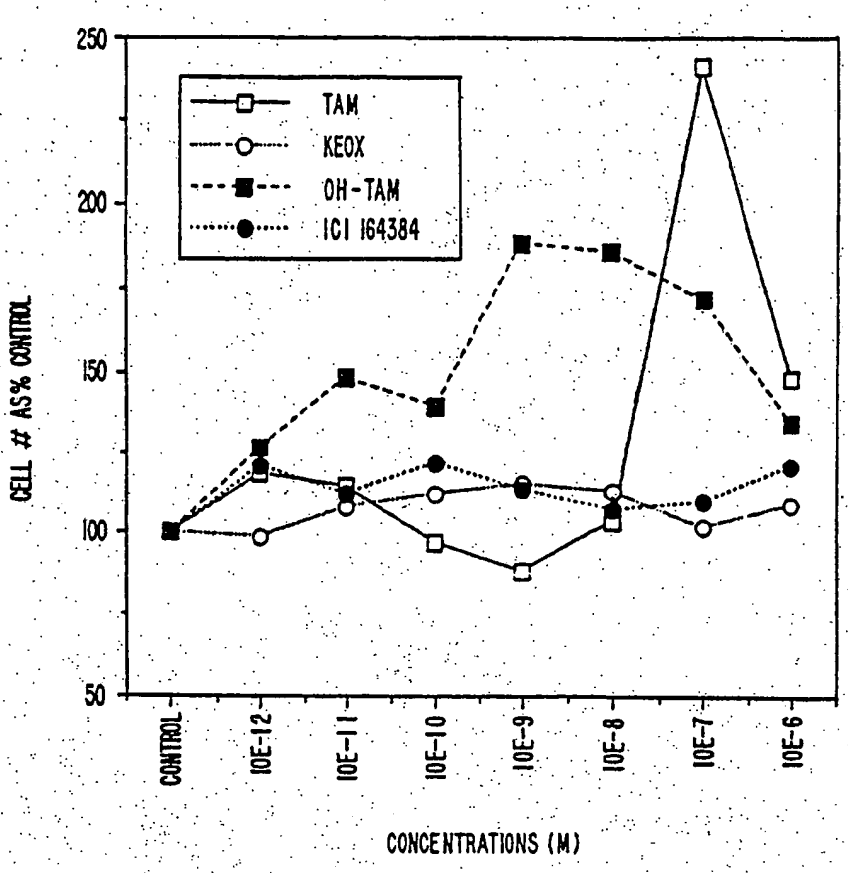
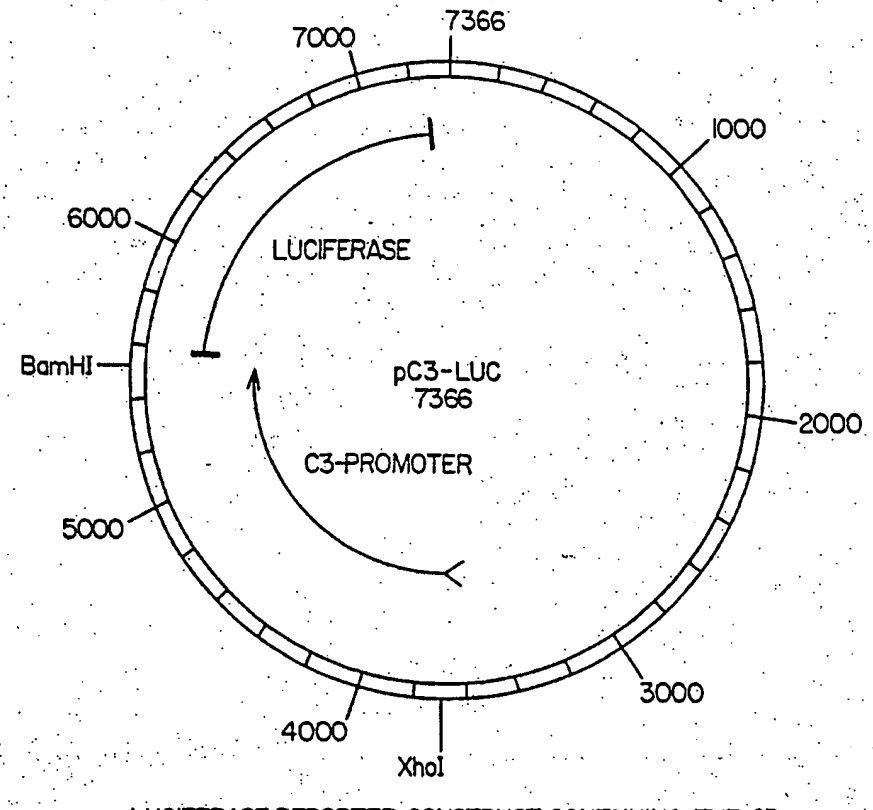

250
200
150
100
50
CELL # AS% CONTROL
TAM
KEOX
OH-TAM
ICI 164384
CONTROL
10E-12
10E-11
10E-10
10E-9
10E-8
10E-7
10E-6
CONCENTRATIONS (M)

FIG. 10.



LUCIFERASE REPORTER CONSTRUCT CONTAINING THE C3 PROMOTER FROM -1807 TO +58

FIG. 11.

INTERNATIONAL SEARCH REPORT

Int ational application No.
PCT/US94/03795

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :C12Q 1/68, 1/00; G01N 33/53, 33/566; C12N 15/00
US CL : 435/6, 7.1, 172.3; 436/501
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.1, 172.3; 436/501

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG
search terms: TAF1, TAF2, estrogen receptor, agonist, liver, hepatocyte, C3 promoter, keoxifene

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,071,773 (EVANS ET AL) 10 December 1991, see entire document.	1-9
Y	EMBO Journal, Volume 11, issued 1992, P. S. Danielian et al, "Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors", pages 1025-1033, see entire document.	1-9
Y	Molecular and Cellular Endocrinology, Volume 34, issued 1985, "J. Simard et al, "Keoxifene shows pure antiestrogen activity in pituitary gonadotrophs", pages 141-144, see entire document.	6, 9

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
15 JUNE 1994	JUL 08 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer D. Kuyza for ELIZABETH C. KEMMERER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

Int ational application No.
PCT/US94/03795

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-9

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Int. ational application No.
PCT/US94/03795

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-9, drawn to a method for screening receptor agonists
II. Claims 10-16, drawn to a method of treating patients with estrogen related diseases
Inventions I and II constitute separate general inventive concepts. The inventions appear to be related in that the agonists identified by the method of Invention I may be used in the method of treating disease of Invention II. However, the Inventions are separate inventive concepts in that the method of treating disease of Invention II can be practiced with compounds identified or prepared in ways other than the screening method of Invention I, such as by organic synthesis. Also, the keoxifene related compounds required by Invention II are not required by Invention I.

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